JOURNAL OF VIROLOGY, July 1997, p. 5295–5303 Vol. 71, No. 7
0022-538X/97/$04.00+0

Characterization of Humoral and CD4$^+$ Cellular Responses after Genetic Immunization with Retroviral Vectors Expressing Different Forms of the Hepatitis B Virus Core and e Antigens†

MATTI SÄLLBERG,[1,2]* KAY TOWNSEND,[2] MARGARET CHEN,[1] JOANNA O'DEA,[2] THERESA BANKS,[2] DOUGLAS J. JOLLY,[2] STEVEN M. CHANG,[2] WILLIAM T. LEE,[2] AND DAVID R. MILICH[1]

Department of Molecular Biology, Scripps Research Institute, La Jolla, California 92037,[1] *and Chiron Technologies Center for Gene Therapy, San Diego, California 92121*[2]

Received 27 January 1997/Accepted 30 March 1997

The humoral and CD4$^+$ cellular immune responses in mice following genetic immunization with three retroviral vectors encoding different forms of hepatitis B virus core antigen (HBcAg) and e antigen (HBeAg) were analyzed. The retroviral vectors induced expression of intracellular HBcAg (HBc[3A4]), secreted HBeAg (HBe[5A2]), or an intracellular HBcAg-neomycin phosphoryltransferase fusion protein (HBc-NEO[6A3]). Specific antibody levels and immunoglobulin G isotype restriction were highly dependent on both the host major histocompatibility complex and the transferred gene. Humoral and CD4$^+$ cellular HBcAg and/or HBeAg (HBc/eAg)-specific immune responses following retroviral vector immunization were of a lower magnitude but followed the same characteristics compared with those after immunization with HBc/eAg in adjuvant. Two factors influenced the humoral responses. First, in vivo depletion of CD8$^+$ cells in HBc-NEO[6A3]-immunized *H-2^k* mice abrogated both HBcAg-specific antibodies and in vitro-detectable cytotoxic T lymphocytes. Second, priming of *H-2^b* mice with an HBc/eAg-derived T-helper (Th) peptide in adjuvant prior to retroviral vector immunization greatly enhanced the HBc/eAg-specific humoral responses to all three vectors, suggesting that insufficient HBc/eAg-specific CD4$^+$ Th-cell priming limits the humoral responses. In conclusion, direct injection of retroviral vectors seems to be effective in priming HBc/eAg-specific CD8$^+$ but comparatively inefficient in priming CD4$^+$ Th cells and subsequently specific antibodies. However, the limited HBc/eAg-specific CD4$^+$ cell priming can effectively be circumvented by prior administration of a recombinant or synthetic form of HBc/eAg in adjuvant.

The hepatitis B virus (HBV) core and e antigens (HBcAg and HBeAg, respectively) are important immunological targets in the HBV-infected host. Both proteins are encoded by the pre-C/C gene and therefore have a common sequence of 149 to 151 amino acids (aa) (39). During the viral life cycle two different mRNAs are transcribed. A longer mRNA starting at the first ATG of the pre-C/C gene is translated into a p25 precursor protein (1a). The p25 is translocated to the endoplasmic reticulum, where the carboxy-terminal 33 to 35 aa and the amino-terminal 19 aa are enzymatically removed (27). The mature protein containing a 9-aa amino-terminal precore signal sequence is then secreted into the circulation in a mono- or dimeric form termed HBeAg (27). A shorter mRNA initiated from the second ATG is translated into the 183-aa HBcAg which assembles into the regularly structured nucleocapsid of HBV (4). The two proteins are antigenically distinct on a B-cell level (5, 6, 9, 29, 31, 32), whereas they are cross-reactive on a T-cell level (22, 23). Thus, despite the high sequence homology between HBeAg and HBcAg, they are completely different proteins with distinct functions in the viral life cycle. It has been proposed that the nonstructural HBeAg acts as a tolerogen in utero and promotes chronicity after neonatal infection (19). In contrast, 180 to 240 copies of the structural HBcAg protein constitute the viral capsid encapsidating the pregenome from which the partially double-stranded HBV genome is completed (4, 39).

During recent years research has highlighted the importance of the immune response to HBcAg and/or HBeAg (HBc/eAg). During acute HBV infection both cytotoxic T cells (CTL) and T-helper (Th) cells specific for HBc/eAg can be detected in the circulation of the infected host (7). In contrast, HBc/eAg-specific CTL and Th-cell activity is not readily detected in chronic HBV infection, except during exacerbations or seroconversion from HBeAg to antibodies to HBeAg (anti-HBe) (11, 41). During chronic asymptomatic HBV infection, there are often no or very few serologic signs of an active immunity against HBV (15, 16).

Treatment of chronic HBV infection has been most effective using alpha interferon (IFN-α), which may induce clearance or seroconversion in 20 to 30% of infected patients. Recent studies suggests that one important factor in the induced or spontaneous seroconversion from HBeAg to anti-HBe is the activation of HBc/eAg-specific T cells, predominantly Th cells (11, 14).

New types of viral therapeutics have included the development of vehicles for gene delivery such as naked DNA (17, 35) and retroviral vectors (10, 13). A major strength of this mode of antigen delivery is that the endogenously produced antigens prime antigen-specific CTL, which are less efficiently evoked with exogenous antigens in adjuvant. It would also be desirable if techniques for gene transfer which would enhance all branches of the host immune system using the endogenous production of the antigen could be developed. However, today very little is known about the effectiveness of genetic immunization in eliciting a multifunctional immune response including

* Corresponding author. Present address: Division of Clinical Virology, F 68, Karolinska Institutet at Huddinge University Hospital, S-141 86 Huddinge, Sweden. Phone: 46-8-5858 79 39. Fax: 46-8-5858 79 33. E-mail: masa@vird01.hs.sll.se.

† Paper 10081-MB from the Scripps Research Institute.

antigen-specific CTL, Th-cell priming, and antibody production. We have therefore developed three retroviral vectors which encode three different forms of HBc/eAg and characterized the humoral responses that they induce in inbred mice. We observed that the humoral responses are highly dependent on the combination of the molecular form, the subcellular localization of the expressed protein, and the major histocompatibility complex (MHC) of the responding strain. Furthermore, we found that a major limiting factor inherent in the retroviral vectors in inducing humoral responses is an inefficiency in priming HBc/eAg-specific Th-cell function compared to protein in adjuvant immunization.

MATERIALS AND METHODS

Inbred mice. Inbred B10, *H-2* congenic (B10 [*H-2*^{b}], B10.D2 [*H-2*^{d}], B10.M [*H-2*^{f}], B10.BR [*H-2*^{k}], B10.P [*H-2*^{p}], and B10.S [*H-2*^{s}]) and C3H/He (*H-2*^{k}) mice were obtained through the breeding colony at the Scripps Research Institute. All mice were immunized at 4 to 8 weeks of age.

Retroviral vectors encoding different forms of HBc/eAg. Three retroviral vectors were produced by using different versions of the HBV pre-C/C gene. The first, containing the complete C gene, encodes a native-like form of HBcAg (HBc[3A4]). A second retroviral vector contains both the pre-C and the C genes whereby the secretory HBeAg is expressed upon transduction (HBe[5A2]). A third vector encodes an in-frame fusion protein between the complete HBcAg sequence and a 10-aa deletion variant of neomycin phosphoryltransferase (HBc-NEO[6A3]). The design, construction, and production of these vector have previously been described in detail (40).

Histology. Producer cell lines were grown to confluence on tissue culture slides. Cells were fixed by acetone and air dried. Cells were stained by hematoxylin and then assayed for HBc/eAg expression with a peroxidase-labelled rabbit anti-HBc/eAg antibody (Dako Corp., Carpinteria, Calif.).

Recombinant antigens and synthetic peptides. Full-length recombinant HBcAg (rHBcAg) encompassing residues 1 to 183 was produced in *Escherichia coli* as previously described (37). This protein assembles into particles with a diameter of 27 nm. A truncated form of HBcAg lacking the 34 carboxy-terminal residues of HBcAg and forming particles at pH 7.2 (HBeAg-7.2) but not at pH 9.6 (HBeAg-9.6) was also *E. coli* derived (22). HBeAg-9.6 exhibits HBeAg-specific antigenicity when used as a solid-phase antigen (22). An alkylated monomeric form of HBeAg (P16) contains residues 1 to 150 of HBc/eAg (37). An additional recombinant form of HBeAg containing nine residues of precore and the 150 first residues of HBcAg was designated PCe (37).

Synthetic peptides corresponding to the HBcAg sequence (*ayw* and/or *adw*) were produced by solid-phase peptide synthesis based on 9-fluorenylmethoxycarbonyl protection on the α-amino group by using manual (8, 33) or automated (Milligen 9050 Plus; Millipore, Bedford, Mass.) procedures. All peptides were analyzed by reversed-phase high-performance liquid chromatography. The sequences of the peptides have been described previously (29, 32).

Retroviral vector and protein immunizations. All retroviral vectors were directly injected in the hind footpads or intramuscularly (i.m.) at volumes of 50 to 100 μl with a 30-gauge needle. Recombinant proteins and synthetic peptides were emulsified in Freund's complete or incomplete adjuvant and were injected subcutaneously (s.c.) in the hind footpads or intraperitoneally (i.p.).

Enzyme immunoassays (EIAs). Expression of HBcAg and HBeAg by the retroviral vector producer cell lines was characterized by a commercially available assay (Sorin Biomedica, Saluggia, Italy). The amounts of HBc/eAg were quantified with rHBcAg as a standard.

Total immunoglobulin G (IgG) and the IgG subclass distribution of anti-hepatitis B core (HBc) and anti-HBe antibodies were determined as previously described by using rHBcAg or HBeAg-9.6 as the solid-phase ligand at 5 or 1 μg/ml, respectively (20, 23, 24). To determine the specificity of the induced antibodies, a limiting amount of antigen was used on the solid phase, 0.5 μg of rHBcAg per ml and 0.1 μg of HBeAg-9.6 per ml. Otherwise, the assays were performed as previously described (20, 23, 24).

Proliferation and cytokine assays. Groups of two to four mice were injected with rHBcAg or PCe emulsified in Freund's complete adjuvant i.p. or s.c. in the hind footpads. Nine to 11 days later the mice were sacrificed, and the spleens or draining lymph nodes (LNs) were harvested. Also, groups of two to four mice were primed, and boosted 3 to 5 days later, with the retroviral vectors in the hind footpads (50 μl) or i.m. (100 μl). The spleens and LNs of these mice were harvested 6 to 7 days after the booster dose. Single-cell suspensions were prepared in Click's medium (3) and plated in microplates at 6×10^5 cells per well for proliferation assays and at 8×10^5 cells per well for cytokine assays. Supernatants were removed at 24 h for determination of interleukin-2 (IL-2), and at 48 h for determination of IL-4 and IFN-γ. The plates for T-cell proliferation were incubated for 96 h with the addition of 1 μCi of [^{3}H]thymidine (Amersham, Buckinghamshire, United Kingdom) for the 16 last h. The labelled cells were harvested onto cellulose filters and quenched, and the level of [^{3}H]thymidine incorporation was determined with a liquid scintillation β-counter.

The presence of cytokines in culture supernatants was determined as previously described (24–26). In brief, the presence of IL-2 in supernatants was determined by proliferation of the IL-2-sensitive NK′A cell line. The presence of IL-4 was determined by proliferation of the IL-4-sensitive CT4.S cell line, and the presence of IFN-γ was determined by a sandwich EIA (Pharmingen, San Diego, Calif.) (24–26).

Detection of cytokine (IL-2, IL-4, IL-10, and IFN-γ) mRNA and β-actin expression was performed by a reverse transcription-PCR. In brief, 2×10^6 cells were cultured in the absence or presence of 5 μg of rHBcAg for 36 h in 1 ml of Click's medium. One microgram of the total RNA, extracted with TRIzol Reagent (GIBCO BRL, Gaithersburg, Md.), was transcribed to cDNA by using Moloney murine leukemia virus reverse transcriptase (GIBCO BRL) and oligo(dT)$_{12–18}$ according to the manufacturer's recommendation. The cDNA was amplified with *Taq* polymerase (Promega, Madison, Wis.) by using primers designed to amplify different cytokine mRNAs (Stratagene, La Jolla, Calif.). The presence of an amplicon was determined by gel electrophoresis and ethidium bromide staining.

RESULTS

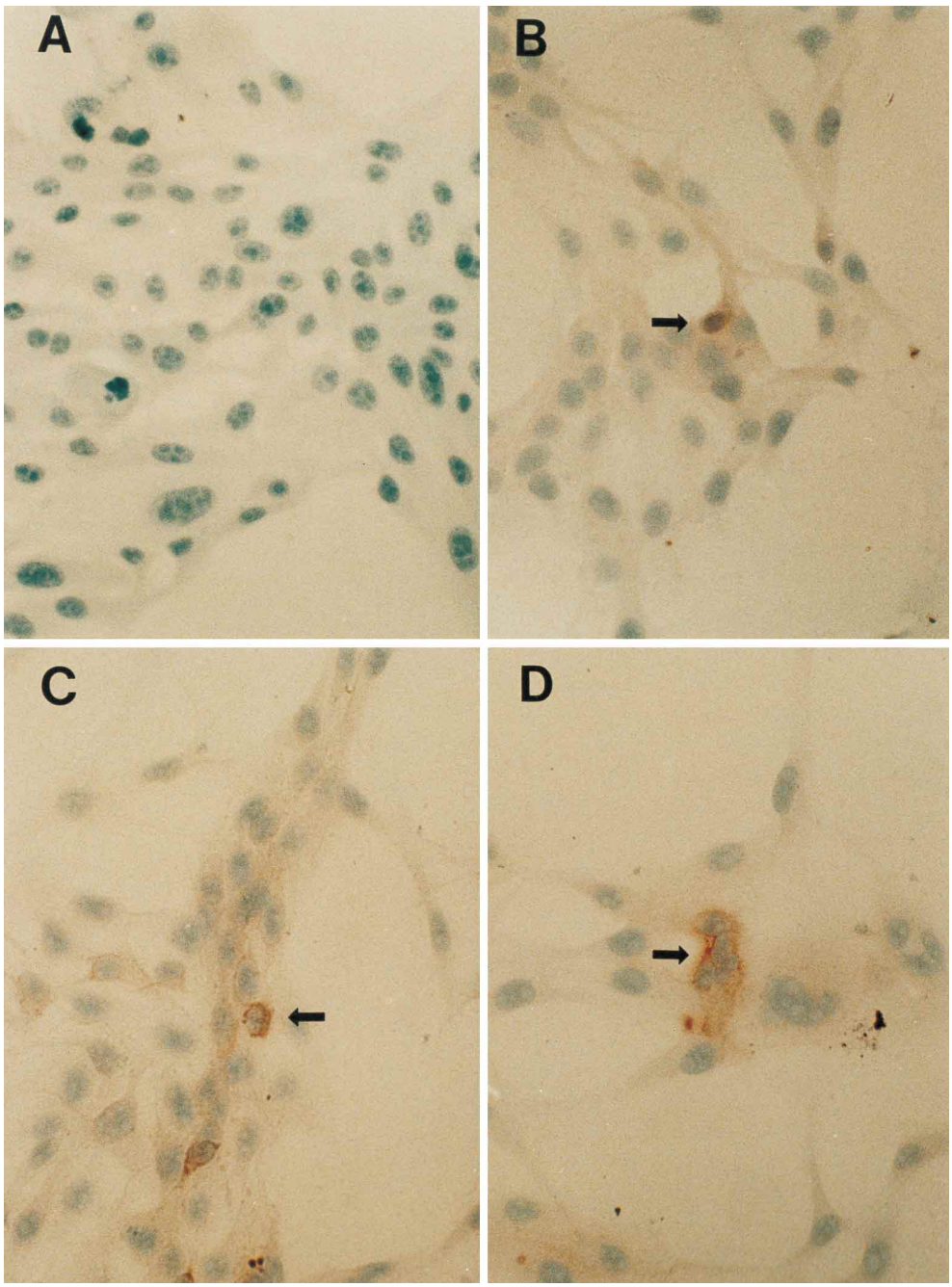
Cellular localization of the HBc/eAg proteins. Staining of expressed HBc/eAg in the DA producer cell lines for the gene products of the three different retroviral vectors indicates that the translated gene products localize to different compartments (Fig. 1). The HBc[3A4] vector encodes a native-like form of HBcAg which is present in both the cytoplasm and the nucleus (Fig. 1). This is consistent with the observed 21-kDa molecular mass in transduced cells (40) and with observations from HBV-infected humans (2, 28). In contrast, the HBe[5A2] retroviral vector-producing cells secrete HBeAg into the culture supernatant (Fig. 1). However, a retained form can be detected in granules in the cytoplasm and most likely is the p25 precursor protein, since a 25-kDa protein with HBc/eAg antigenicity could be detected by Western blot (Fig. 1 and unpublished data). The HBc-NEO[6A3] fusion protein appears to be restricted to the cytoplasmic compartment and does not show a clear nuclear staining as observed for native HBcAg (Fig. 1). This fusion protein shows a molecular mass of 50 kDa in Western blot (40).

Irrespective of the cell line used, proteins encoded by the three different retroviral vectors are consistently located in either the intracellular or both intra- and extracellular compartments (Fig. 1 and data not shown). In analysis of the HBc/eAg expression of the DA vector producer cell clones by EIA (Sorin), both the HBc[3A4] and the HBc-NEO[6A3] retroviral vectors produce mainly intracellular proteins, since they are detected in the cell lysate at levels 10 to 100 times higher than in the culture supernatants. In contrast, the HBe[5A2] retroviral vector produces both the secreted mature HBeAg and the intracellular p25 precursor, as detected by histological staining (Fig. 1). This further confirms the different subcellular localization of the proteins expressed by the three retroviral vectors independent of the transduced target cell.

Antibody responder status in relation to MHC. The three retroviral vectors were injected i.m. at weeks 0, 2, and 4 into panels of B10, *H-2* congenic mice. The mice were bled every second week for 8 weeks. As shown in Fig. 2, the antibody responder or nonresponder status is *H-2* restricted after injection with the different HBc/eAg retroviral vectors.

The best antibody responders to the HBc[3A4] retroviral vector were found to be the *H-2*^{b}, *H-2*^{f}, and *H-2*^{p} haplotype mice, which all developed low levels of anti-HBc 4 to 6 weeks after the first injection. No B10.BR (*H-2*^{k}) or B10.S (*H-2*^{s}) mice produced anti-HBc (Fig. 2).

With the HBe[5A2] retroviral vector, a rapid anti-HBe response was detected 2 weeks after the first immunization in the *H-2*^{b}, *H-2*^{f}, *H-2*^{p}, and *H-2*^{s} haplotype mice. The *H-2*^{d} mice developed low levels of anti-HBe only after the third injection, whereas none of the three B10.BR (*H-2*^{k}) mice developed



Detection of HBc/eAg by EIA (Sorin)

Ag source	A) DA	B) DA-HBc[3A4]	C) DA-HBc[5A2]	D) DA-HBc/neo[6A3]
Supernatant	<0.1 ng/ml	1.5 ng/ml	706 ng/ml	5.9 ng/ml
Lysate	<0.1 ng/ml	617 ng/ml	904 ng/ml	551 ng/ml

FIG. 1. Hematoxylin and immunostaining of the DA–β-galactosidase (a), DA-HBc[3A4] (b), DA-HBe[5A2] (c), and DA–HBc-NEO[6A3] (d) producer cell lines for HBcAg and HBeAg using peroxidase-labelled rabbit anti-HBc/e (Dako). Also indicated is the detection of HBcAg and HBeAg in the supernatants and cell lysates of the DA producer cell lines. One hundred microliters of supernatant or 10^6 lysed cells were assayed together with a standard dilution of HBcAg in a commercial HBeAg EIA (Sorin Biomedica). Values are given as nanograms of HBc/eAg per milliliter.

anti-HBe, similar to the low-responder or nonresponder status observed for the HBc[3A4] retroviral vector.

The humoral responses to the HBc-NEO[6A3] vector were significantly different from those to the HBc[3A4] vector despite the similar cellular localization (Fig. 2). Within 4 to 6 weeks after the first injection, only *H-2*^{k} haplotype mice immunized with the HBc-NEO[6A3] vector developed antibodies to HBcAg and/or HbeAg (anti-HBc/e) (Fig. 2). Low levels of

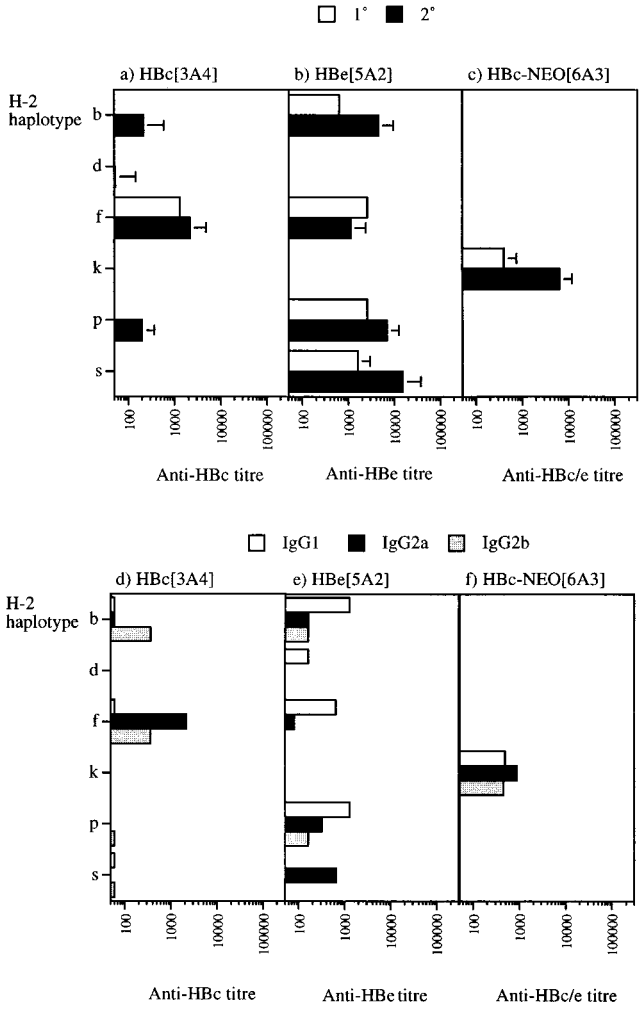


FIG. 2. (a to c) Humoral responses to retrovector immunization in B10 congenic mice of six haplotypes. Mice were injected at week 0 and week 2 with 200 μl of retrovector i.m., and each bar represents bleeds at week 2 (1°) and week 4 (2°). All sera were tested at six fourfold dilutions starting at 1:40. The mice immunized with the HBc[3A4] vector were analyzed with HBcAg as the solid-phase antigen, whereas HBeAg was used for the other two groups of mice (HBe[5A2] and HBc-NEO[6A3]). Each value represents a mean of the endpoint titers of a group of three mice. A reaction was considered positive if it exceeded the optical density of a nonimmunized mouse by three times. (d to f) Isotypes of anti-HBc and anti-HBe at 6 weeks following first retrovector immunization. Each value represents the endpoint titer of pooled sera from a group of three mice. Endpoint titers were determined as described for total IgG.

anti-HBc/e were detected in other haplotypes at week 8, 2 weeks after the third injection (data not shown).

Antibody specificity and IgG isotypes induced by immunization with retroviral vectors. All sera collected from the three B10, *H-2* congenic panels were evaluated to further confirm the specificity for HBcAg and/or HBeAg. The assays were designed with a suboptimal amount of antigen on the solid phase to better discriminate between anti-HBc and anti-HBe specificity. The HBc[3A4] vector clearly induced antibodies with HBcAg specificity in the $H\text{-}2^b$ and $H\text{-}2^f$ haplotype mice (data not shown). In contrast, the HBe[5A2] vector, which upon transduction induces secretion of HBeAg, clearly elicited anti-HBe in the $H\text{-}2^b$, $H\text{-}2^f$, $H\text{-}2^p$, and $H\text{-}2^s$ haplotype mice. Therefore, both the HBc[3A4] and the HBe[5A2] vectors induced antibody specificities similar to those observed in HBV-infected humans (5, 9, 34).

Not surprisingly, the HBc-NEO[6A3] fusion retroviral vector induced antibodies which were highly cross-reactive against both rHBcAg and HBeAg-9.6. They did not show the classical anti-HBc-like pattern, and the reactivity was more indicative of an anti-HBe-like recognition. This is most likely explained by the nature of the HBc-NEO[6A3] fusion protein, which probably does not assemble into capsids, which is an important structural feature for the antigenicity of native HBcAg (29, 37).

With respect to the HBc[3A4] retroviral vector, all IgG isotypes of anti-HBc were detected except for IgG3 (Fig. 2). In the $H\text{-}2^b$ and $H\text{-}2^p$ haplotypes IgG2b was predominant, whereas IgG2a was predominant in $H\text{-}2^f$ mice. With rHBcAg in the adjuvant as the immunogen, IgG2b is most often the dominant isotype in $H\text{-}2^b$, $H\text{-}2^k$, and $H\text{-}2^s$ haplotype mice (reference 20 and unpublished observations).

The response to the HBe[5A2] retroviral vector showed an MHC-dependent IgG isotype distribution of the anti-HBe antibodies produced (Fig. 2). The anti-HBe IgG1 subclass was predominant in the $H\text{-}2^b$, $H\text{-}2^d$, $H\text{-}2^f$, and $H\text{-}2^p$ haplotype mice, whereas anti-HBe IgG2a was predominant in the $H\text{-}2^s$ haplotype mice and in one $H\text{-}2^k$ mouse producing antibodies to the HBe[5A2] retroviral vector (data not shown). Previously the $H\text{-}2^b$ haplotype has been shown to be a predominantly Th2-like responder to HBc/eAg and the $H\text{-}2^s$ haplotype has been shown to be a mainly Th1-like responder (24). The present data on the isotype distribution using the HBe[5A2] retroviral vector correlate well to the reported influence of Th-cell phenotype on IgG subclass distribution observed following immunization with recombinant HBc/eAg (25, 38).

With respect to the HBc-NEO[6A3] vector, all IgG isotypes with the exception of IgG3 were produced in $H\text{-}2^k$ haplotype mice (Fig. 2). In the other haplotype mice mainly anti-HBc/e IgG1 could be detected after a third injection (data not shown).

HBc/eAg-specific Th-cell priming induced by retroviral vector immunization. To directly compare the level of Th-cell priming by the HBc/eAg-encoding retroviral vectors to the level of Th-cell priming induced by recombinant HBc/eAg (rHBc/eAg) in adjuvant, groups of three to four C3H/He ($H\text{-}2^k$) mice were immunized in the footpads. As shown in Fig. 3, the level of Th-cell priming is more efficient by rHBcAg-complete Freund's adjuvant immunization than by either HBc [3A4] or HBc-NEO[6A3] immunization. Neither of the two retroviral vectors is able to prime Th cells which are efficiently recalled by the synthetic Th-cell site defined by residues 111 to 130 of HBc/eAg. These data indicate that the limited level of HBc/eAg Th-cell priming may explain the comparatively weak humoral responses following retroviral vector immunization. Moreover, proliferation could be blocked by addition of monoclonal anti-CD4 antibody (GK1.5; ATCC TIB207) but not by monoclonal anti-CD8 antibody (2.43; ATCC TIB210) in the in vitro cultures (data not shown), confirming $CD4^+/CD8^-$ cells as the proliferating phenotype.

The cytokine phenotype of the polyclonal HBc/eAg-specific Th-cell response following immunization with the HBc-NEO[6A3] retroviral vector was evaluated. Groups of three B10.BR ($H\text{-}2^k$) and B10.S ($H\text{-}2^s$) mice were primed and boosted with a total dose of 200 μl of HBc-NEO[6A3] in the hind footpads. Both splenic and LN HBc/eAg-specific Th cells were analyzed with respect to the recall cytokine profiles. In vivo-primed HBc/eAg-specific Th cells from both haplotypes predominantly produced IL-2 and IFN-γ on recall with rHBcAg (Fig. 4). The cytokine profiles were reiterated when the cytokine mRNA expression was determined by PCR (data not

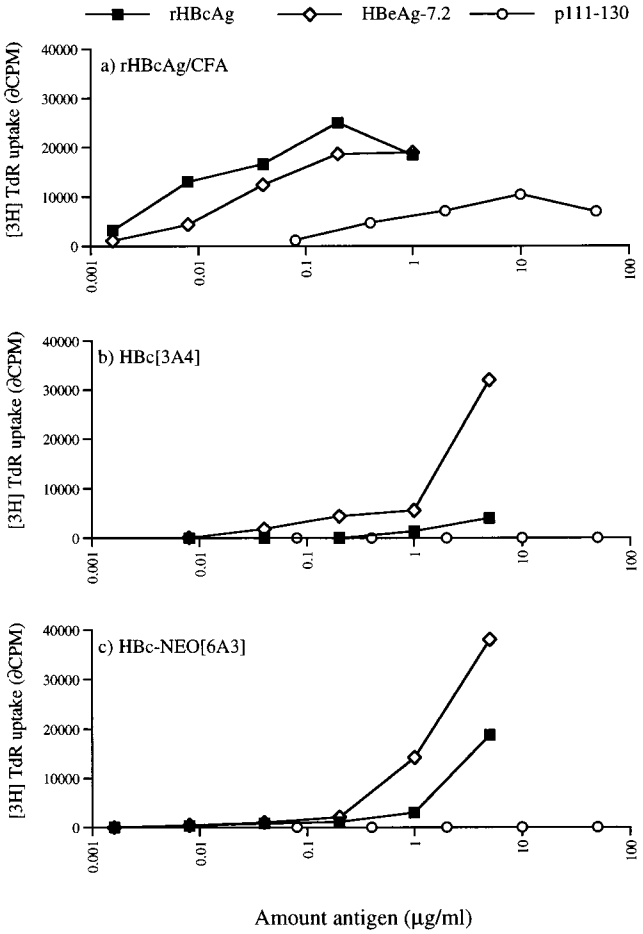


FIG. 3. Comparison of HBc/eAg T-cell priming efficiency by s.c. injections in the hind footpads of 10 μg of rHBcAg in complete Freund's adjuvant (CFA) (a), 2 × 100 μl of HBc[3A4] (b), or 2 × 100 μl of HBc-NEO[6A3] (c). Groups of three C3H (*H-2*^{k}) mice were primed (retroviral vector-immunized mice were boosted 5 days later) and sacrificed 9 to 11 days later. Draining LNs were harvested, and single-cell suspensions were cultured for 96 h in the absence or presence of the indicated antigens. Values are given as the counts per minute with antigen with subtraction of the mean counts per minute of wells without antigen (δCPM). [3H] TdR, [^{3}H]thymidine.

shown). This indicates that predominantly Th1-like HBc/eAg-specific Th cells are induced by immunization with the HBc-NEO[6A3] retroviral vector, as previously suggested by the efficient production of anti-HBc/e IgG2a observed in *H-2*^{k} mice (Fig. 2). However, the low level of HBc/eAg Th-cell priming induced by either of the retroviral vectors may by itself exclude the activation of possibly low-affinity HBc/eAg-specific Th2-like cells (25). Although the *H-2*^{s} haplotype has been shown to be a predominantly Th1-like responder to HBc/eAg, splenic IL-4 can usually be detected at low levels following immunization with rHBcAg in adjuvant (24). This may further indicate that the lack of IL-4 production is an additional sign of a comparatively low level of Th-cell priming following retroviral vector immunization. Further evidence for the comparatively low Th-cell priming might be the absence of antibodies to the HBc-NEO[6A3] retroviral vector in any haplotype other than *H-2*^{k}. Interestingly, despite the ability of the HBc-NEO[6A3] retroviral vector to induce HBc/eAg-specific Th-cells in *H-2*^{s} mice after two injections, antibodies were not detectable until a third injection was given (data not shown). This may be due to the intracellular localization of this translation product.

To further characterize the phenotype of the primed T cells, additional studies were performed using the HBe[5A2] retroviral vector to immunize mice of the *H-2*^{b} and *H-2*^{s} haplotypes, since both these haplotypes were high antibody responders to this vector. Previous studies using immunization with rHBcAg in adjuvant have shown that in *H-2*^{b} haplotype mice a predominantly Th2-like HBc/eAg-specific splenic response is observed, whereas *H-2*^{s} haplotype mice are predominantly Th1-like responders (24). Groups of four *H-2*^{b} and *H-2*^{s} mice were primed and boosted i.m. with 2 × 10^7 CFU of the HBe[5A2] retroviral vector. Both LN and splenic proliferative responses were detected in both strains (Fig. 5). Only low levels of IL-2 could be detected in culture supernatants (data not shown). The phenotype of in vivo-primed and in vitro-recalled HBc/eAg-specific splenic T cells was therefore determined by detection of cytokine mRNA expression by PCR. As shown in Fig. 5, IL-2 and IL-4 mRNA expression is recalled by HBcAg in B10 splenocytes, whereas mainly IL-2 and IFN-γ mRNA expression is detected in *H-2*^{s} splenocytes. Since the Th-cell

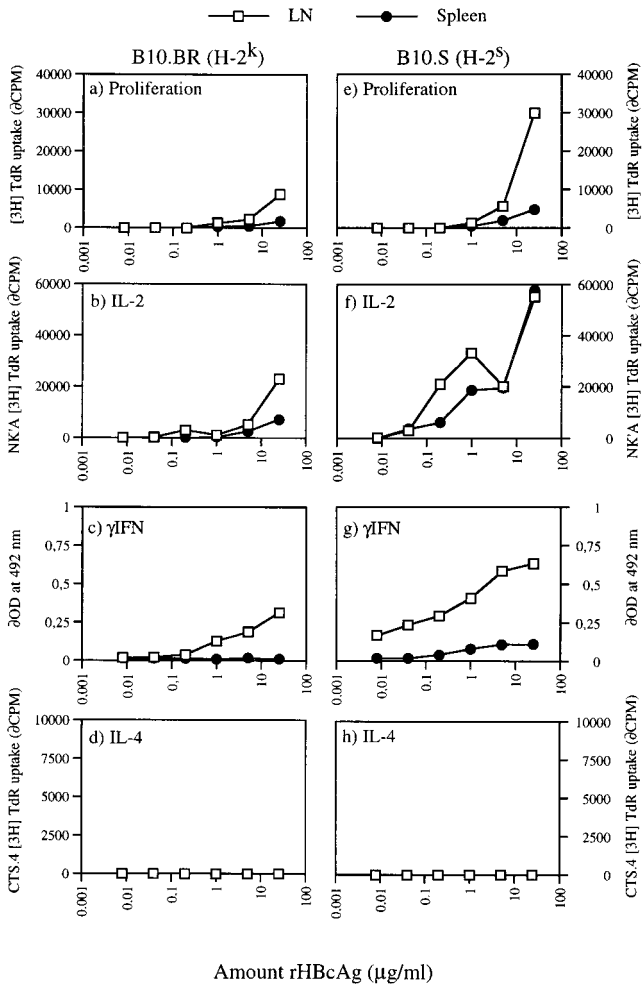


FIG. 4. Cytokine profiles of B10.BR (*H-2*^{k}) (a to d) and B10.S (*H-2*^{s}) (e to h) T cells primed by the HBc-NEO[6A3] retroviral vector. Groups of three mice were primed and boosted 3 days later with 2 × 50 μl of vector in the hind footpads. Seven days later splenic and LN T cells were harvested and cultured in the absence or presence of rHBcAg for 24 to 96 h as described in Materials and Methods. [3H] TdR, [^{3}H]thymidine.

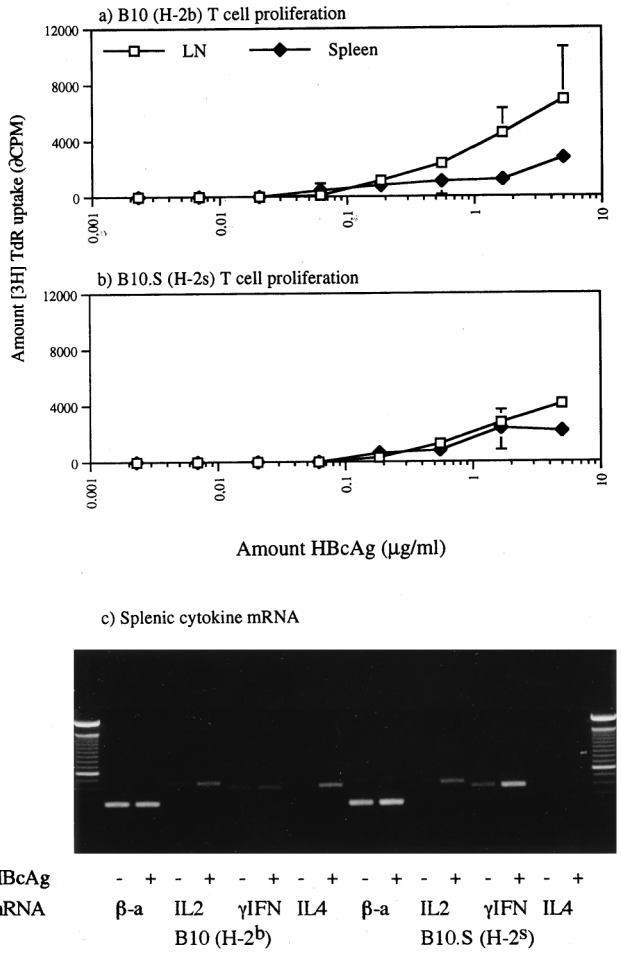


FIG. 5. Proliferative (a and b) and cytokine (c) responses to rHBcAg of B10 ($H\text{-}2^b$) and B10.S ($H\text{-}2^s$) LN and splenic T cells following immunization with the HBe[5A2] retroviral vector. Mice were primed and boosted in the hind footpads as described in the legend to Fig. 3. Proliferation was determined at 96 h, and cytokine mRNA was extracted from splenic 48-h cultures as described in Materials and Methods.

cytokine phenotype has been found to influence IgG heavy-chain switching (38), the present cytokine data correlate well with the IgG isotypes of anti-HBe detected in HBe[5A2]-immunized $H\text{-}2^b$ (predominantly IgG1) and $H\text{-}2^s$ (predominantly IgG2a) mice. Altogether, despite less efficient Th-cell priming, immunization with the HBe[5A2] retroviral vector primes similar, MHC-dependent, Th-cell cytokine phenotypes, as does immunization with rHBc/eAg in adjuvant.

To exclude the influence of exogenous HBc/eAg carried over from the DA producer cell clone supernatants, control experiments were performed. The formulated HBe[5A2] retroviral vector preparation contained levels of HBc/eAg (80 ng/ml) 40- to 100-fold higher than the other vector preparations as determined by HBc/eAg EIA (Sorin). Therefore, a portion of the HBe[5A2] retroviral vector preparation was heat inactivated for 30 min at 56°C, which does not affect the antigenicity of HBeAg, prior to injection in two $H\text{-}2^b$ mice. Two $H\text{-}2^b$ mice immunized with untreated HBe[5A2] served as controls. On in vitro recall, only splenocytes from the B10 mice immunized with noninactivated HBe[5A2] showed proliferation and IL-2 production (data not shown). Thus, a total dose of 400 μl of heat-inactivated HBe[5A2] retroviral vector (i.e., 32 ng of HBeAg) is insufficient to prime an in vitro-detectable Th-cell response. Moreover, anti-HBe was not detected in the mice given the heat-inactivated retroviral vector but was observed in both mice given the untreated vector (mean titer, 960 ± 452). This confirms that possible remnants of exogenous HBc/eAg do not induce detectable responses.

Factors limiting humoral responses following retroviral vector immunization. Experiments were performed to determine whether the amount of expressed antigen available for HBc/eAg-specific B cells or the priming event of HBc/eAg-specific Th cells limits the magnitude of the humoral response following retroviral vector immunization. These studies were performed with the $H\text{-}2^b$ haplotype since previous reports have identified a synthetic T-cell site, residues 129 to 140 of HBc/eAg, which effectively primes helper T cells but which does not induce antibodies cross-reactive with HBeAg (21). Groups of two to three B10 ($H\text{-}2^b$) mice were primed with 100 μg of the $H\text{-}2^b$-restricted HBc/eAg-specific Th-cell peptide in incomplete Freund's adjuvant 9 days prior to retroviral vector immunization. An equal number of non-peptide-primed vector-immunized B10 mice served as controls.

Independent of the retroviral vector used to immunize $H\text{-}2^b$ mice, it is clear that the amount of antigen expressed in vivo and accessible to B cells is not limiting, since peptide-primed mice (with an excess of HBc/eAg-specific Th cells) showed more rapid, higher-titer, and longer-lived anti-HBc/e responses compared to responses in the control mice (Fig. 6). The mice primed with the Th-cell-specific peptide showed high levels of anti-HBc/e persisting for more than 10 weeks. Obviously, a single injection of the HBe[5A2] retroviral vector does not prime HBc/eAg Th cells as efficiently as a protein or peptide in adjuvant. However, in the presence of a nonlimiting source of HBc/eAg-specific Th cells, the retroviral vectors produce sufficient antigen in vivo to engage B cells and maintain a long-lived humoral response.

Both the HBc[3A4] and HBc-NEO[6A3] retroviral vectors induced lower levels of specific antibodies than the HBe[5A2]

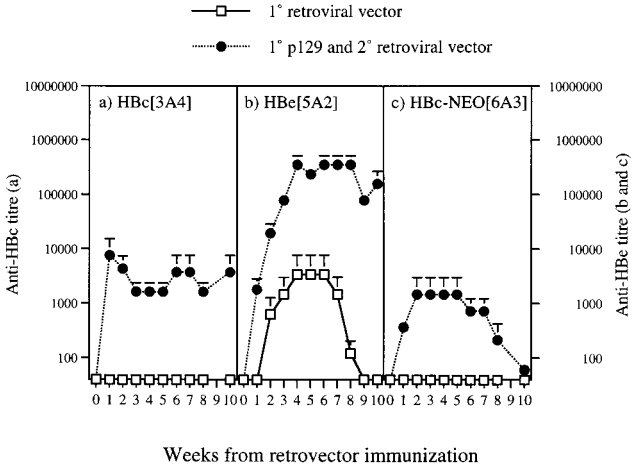


FIG. 6. Antibody responses in B10 ($H\text{-}2^b$) mice following immunization with the HBc[3A4], HBe[5A2], and HBc-NEO[6A3] retrovectors can be enhanced by prior priming with a synthetic Th-cell site corresponding to residues 129 to 140 of HBc/eAg. Groups of two to three mice were primed with 100 μg of peptide in incomplete Freund's adjuvant 9 to 11 days prior to retrovector immunization. The mice were bled prior to retrovector immunization and each week for 6 weeks thereafter. An equal number of mice receiving only the retrovector immunization served as controls. Endpoint titers were determined as described for Fig. 2. Each value represents a mean of the endpoint titers of a group of two to three mice.

retroviral vector in the presence of unlimited HBc/eAg-specific T-cell help (Fig. 6). Since both HBcAg and HBc-NEO proteins are localized mainly to the intracellular compartment, not only the level of Th-cell priming limits these responses but also the amount of antigen readily accessible to B cells is a factor. The persisting anti-HBc levels produced to the HBc[3A4] retroviral vector suggest that low levels of HBcAg continuously leave the cell. This is highly consistent with studies in HBcAg transgenic mice in which the transfer of HBcAg-specific Th cells induces anti-HBc in the absence of liver damage or an exogenous source of HBcAg (18).

Additional experiments were performed to evaluate why only *H-2*^{k} mice produced antibody following immunization with the HBc-NEO[6A3] retroviral vector. Note that only *H-2*^{k} mice are CTL responders following HBc-NEO[6A3] retroviral vector immunization (40). B10.BR (*H-2*^{k}) mice were depleted of CD8$^+$ T cells in vivo by using 0.5 mg of the anti-CD8 monoclonal antibody. In a control group B10.BR mice were given equal doses of rat IgG. Three days after the initial i.p. injection of anti-CD8, both groups were primed by i.m. injection with 2×10^7 CFU of HBc-NEO[6A3]. The mice were boosted 1 week later with an equal dose. The depletion was maintained by weekly injections of 0.1 mg of anti-CD8 throughout. The depletion of CD8$^+$ cells completely abrogated the induction of both HBc/eAg-specific antibodies and CTL 4 weeks after the first immunization, suggesting that HBc/eAg-specific CTL are needed for the release of antigen in sufficient amounts to prime antibody production. In the mock-treated control group, all mice had detectable HBc/eAg-specific antibodies (mean anti-HBc/e titer, 570 ± 321) and CTL (mean lysis of HBcAg-expressing LM cells above background 17.3% ± 3.2% at 100:1 effector/target ratio).

DISCUSSION

Little is known about the behavior of retroviral and DNA-based vectors as immunogens with respect to how efficiently the different branches of the immune system are primed. Several studies have documented the efficiency by which retroviral vectors prime antigen-specific CTL (10, 13, 40). This is, with few exceptions (37), a unique feature of genetic immunizations, since endogenously produced antigen is inevitably introduced into the class I antigen processing pathway. However, with respect to the basis of the humoral and Th-cell responses, the efficacy of genetic immunizations has been less well characterized and explored.

Using the three retroviral vectors encoding different forms of HBc/eAg as immunogens, our study indicated that the form of HBc/eAg, the localization of the expressed protein, and the MHC of the host all have an influence on the immune response. When rHBc/eAg is used in adjuvant as the immunogen, a hierarchy can be found in the level of the humoral and CD4$^+$ cellular responses (23). High humoral responders to rHBcAg are the *H-2*^{d}, *H-2*^{k}, and *H-2*^{s} haplotypes; intermediate responders are the *H-2*^{b} and *H-2*^{f} haplotypes; and *H-2*^{p} is the only low responder (23). This hierarchy was reiterated when the Th-cell priming to the same antigen was determined (23). As shown herein, the same hierarchy could not be observed with retroviral vector immunization with different forms of HBc/eAg. In contrast, the high responders to rHBcAg, the *H-2*^{d}, *H-2*^{k}, and *H-2*^{s} mice, were all poor humoral responders to the HBc[3A4] retroviral vector, whereas the intermediate or low responder *H-2*^{b}, *H-2*^{f}, and *H-2*^{p} haplotype mice were found to be the best humoral responders to the HBc[3A4] retroviral vector.

Furthermore, the good responders to the HBe[5A2] retroviral vector were low or intermediate responders to rHBc/eAg in adjuvant (23), with the exception of the B10.S (*H-2*^{s}) strain. These discrepancies are not easy to explain, since many factors could affect these responses. First, the presence of a CTL response may limit the longevity of the protein expression in the host such as in the *H-2*^{k} haplotype. However, it should be noted that in the *H-2*^{k} haplotype mice HBc/eAg-specific CTL seemed to be necessary for antibody responses to the HBc-NEO[6A3] retroviral vector. This relation may be due to low in vivo expression levels of HBc/eAg and may well change if expression levels are increased. Second, since the hierarchy of the rHBc/eAg-specific response was determined with injections with 1 to 10 μg of antigen, a different hierarchy may be observed from the substantially lower levels of antigen expressed in vivo by the retroviral vectors. The affinity of the trimolecular MHC–peptide–T-cell receptor complex may more profoundly influence the response at these lower doses. Regardless of the reason, it is obvious that this different route of immunization induces a different hierarchy of responder status which is dependent on both the molecular form and localization of the antigen and the host MHC. Again, it cannot be excluded that the hierarchies observed herein will change if the in vivo expression levels of the transferred gene is changed, as suggested in a recent report (12). In fact, we now have preliminary evidence that different types of pretreatment of the injection site before retroviral vector- or DNA-mediated gene transfer enhance levels of expression and the subsequent humoral responses (1). Furthermore, an inherent difference with the retroviral vector gene delivery system is that the retroviral infection or transduction of the target cell may by itself induce a certain type of host immune response and cytokine environment (i.e., IFN-γ) which may influence the overall response to the transferred gene.

In comparison to immunization with recombinant antigens, it is clear that retroviral vector immunization alone is less efficient in inducing humoral responses, with respect to both the levels and the persistence of detectable antibodies. However, despite these obvious differences, the overall characteristics of the responses are similar. The specificities of the induced antibodies were similar to those observed after immunization with recombinant proteins and in natural HBV infection.

We could confirm that the level of Th-cell priming is less efficient following immunization with retroviral vectors compared to that following exogenous protein in adjuvant. However, the two responses are highly similar with respect to the primed Th-cell phenotypes. Throughout this study we have failed to detect Th2-like cytokines in recall culture supernatants. The presence of IL-4-producing HBc/eAg-specific Th cells was detected only by cytokine mRNA expression by PCR. This could indicate that immunization by retroviral vectors preferentially primes Th1-like T cells, similar to observations with naked DNA as the immunogen (30). However, an equally likely explanation is that the T-cell priming by the retroviral vector preferentially primes high-affinity Th1-like cells simply due to the limited amount of antigen expression in combination with the absence of adjuvant. Higher in vivo expression levels of antigen may also prime Th2-like cells. Further studies with other gene delivery systems may resolve this question.

An interesting paradox is the responder or nonresponder status of the *H-2*^{k} haplotype depending on the retroviral vector used for immunization. The *H-2*^{k} haplotype was a nonresponder to the HBc[3A4] and the HBe[5A2] vectors and a responder to the HBc-NEO[6A3] retroviral vector in terms of antibody responses. Also, we found that the humoral response to the HBc-NEO[6A3] vector was dependent on the presence

of CTL, possibly to release sufficient amounts of antigen. Thus, this may imply that depending on the vector construct and the expression levels, CTL is either a limiting or a beneficial factor with respect to the humoral responses. However, peptide priming in CTL nonresponder *H-2*^{b} mice prior to HBc-NEO[6A3] retroviral vector immunization induced a clearly detectable but short-lived humoral response, which suggests that a limited amount of antigen leaves the cell in the absence of CTL. Also, the fusion protein nature of the HBc-NEO[6A3] might influence the HBc/eAg-specific immune response.

The most striking observation was that the limited HBc/eAg-specific Th-cell priming following retroviral vector immunization hampered the humoral responses. Using either the HBc[3A4], the HBe[5A2], or the HBc-NEO-NEO[6A3] retroviral vector, we found that the amount of antigen present in vivo was not sufficient to elicit a high level of Th priming. A prior injection of a synthetic Th-cell site greatly enhanced both the levels and the persistence of anti-HBc and anti-HBe antibody responses in *H-2*^{b} haplotype mice. However, it is unclear whether it is the lack or absence of inflammation or low levels of antigen that limits the Th-cell priming event. Thus, it seems imperative to enhance the Th-priming event during retroviral vector immunizations if humoral responses are desired. Also, this implies that a combination of protein(s) or peptide(s) in adjuvant and retroviral vector- or naked DNA-based immunizations is desired if all branches of the specific immune response are to be effectively primed. Again, we anticipate that this may not be true for all antigen systems and will also be dependent on the expression levels of the vector system used.

In conclusion, retroviral vectors encoding different forms of HBc/eAg can effectively be employed to prime humoral responses. One significant limiting factor for the magnitude of the HBc/eAg-specific humoral response is the level of Th-cell priming. Thus, further studies are needed to determine the nature of the retroviral vector-primed Th-cell population and how it may be possible to enhance the Th-cell priming event. However, for therapeutic purposes it may be safer and even advantageous in some circumstances to elicit Th-cell and CTL priming independently.

ACKNOWLEDGMENTS

This work was supported in part by National Institutes of Health grants AI20720 and AI33562.

REFERENCES

1. **Banks, T., et al.** Unpublished data.
1a. **Bruss, V., and W. H. Gerlich.** 1988. Formation of transmembranous hepatitis B e-antigen by cotranslational in vitro processing of the viral precore protein. Virology **163:**268–275.
2. **Chu, C.-M., C.-T. Yeh, I.-S. Sheen, and Y.-F. Liaw.** 1995. Subcellular localization of hepatitis B core antigen in relation to hepatocyte regeneration in chronic hepatitis B. Gastroenterology **109:**1926–1932.
3. **Click, R. L., and B. J. Alter.** 1972. Immune response in vitro. I. Culture conditions for antibody synthesis. Cell. Immunol. **3:**264–276.
4. **Crowther, R. A., N. A. Kiselev, B. Böttcher, J. A. Berriman, G. P. Borisova, V. Ose, and P. Pumpens.** 1994. Three-dimensional structure of hepatitis B virus core particles determined by electron cryomicroscopy. Cell **77:**943–950.
5. **Ferns, R. B., and R. S. Tedder.** 1986. Human and monoclonal antibodies to hepatitis B core antigen recognise a single immunodominant epitope. J. Med. Virol. **19:**193–203.
6. **Ferns, R. B., and R. S. Tedder.** 1984. Monoclonal antibodies to hepatitis B e antigen (HBeAg) derived from hepatitis B core antigen HBcAg: their use in characterization and detection of HBeAg. J. Gen. Virol. **65:**899–908.
7. **Ferrari, C., A. Penna, A. Bertoletti, A. Valli, A. D. Antoni, T. Giuberti, A. Cavalli, M. A. Petit, and F. Fiaccadori.** 1990. Cellular immune response to hepatitis B virus-encoded antigens in acute and chronic hepatitis B virus infection. J. Immunol. **145:**3442–3449.
8. **Hougthen, R. A.** 1985. General method for the rapid solid-phase synthesis of large numbers of peptides: specificity of antigen-antibody interaction at the level of individual amino acids. Proc. Natl. Acad. Sci. USA **82:**5131.
9. **Imai, M., M. Nomura, T. Gotanda, T. Sano, K. Tachibana, H. Miyamoto, K. Takahashi, S. Toyama, Y. Miyakawa, and M. Mayumi.** 1982. Demonstration of two distinct determinants on hepatitis B e antigen by monoclonal antibodies. J. Immunol. **128:**69–72.
10. **Irwin, M. J., L. S. Laube, V. Lee, M. Austin, S. Chada, C. G. Anderson, K. Townsend, D. J. Jolly, and J. F. Warner.** 1994. Direct injection of a recombinant retroviral vector induces human immunodeficiency virus-specific immune responses in mice and nonhuman primates. J. Virol. **68:**5036–5044.
11. **Jung, M.-C., H. M. Diepolder, U. Spengler, E. A. Wierenga, R. Zachoval, R. M. Hoffman, D. Eichenlaub, G. Frösner, H. Will, and G. R. Pape.** 1995. Activation of a heterogenous hepatitis B (HB) core and e antigen-specific $CD4^+$ T-cell population during seroconversion to anti-HBe and anti-HBs in hepatitis B virus infection. J. Virol. **69:**3358–3368.
12. **Kuhober, A., H. P. Pudollek, K. Reifenberg, F. V. Chisari, H. J. Schlicht, J. Reimann, and R. Schirmbeck.** 1996. DNA immunization induces antibody and cytotoxic T cell responses to hepatitis B core antigen in H-2(B) mice. J. Immunol. **156:**3687–3695.
13. **Laube, L. S., M. Burrascano, C. E. Dejesus, B. D. Howard, M. A. Johnson, W. T. Lee, A. E. Lynn, G. Peters, G. S. Ronlov, K. S. Townsend, et al.** 1994. Cytotoxic T lymphocyte and antibody responses generated in rhesus monkeys immunized with retroviral vector-transduced fibroblasts expressing human immunodeficiency virus type-1 IIIB ENV/REV proteins. Hum. Gene Ther. **5:**853–862.
14. **Marinos, G., F. Torre, S. Chokshi, M. Hussain, B. E. Clarke, D. J. Rowlands, A. L. W. F. Eddelston, N. V. Naoumov, and R. Williams.** 1995. Induction of T-helper cell response to hepatitis B core antigen in chronic hepatitis B: a major factor in activation of the host immune response to the hepatitis B virus. Hepatology **22:**1040–1049.
15. **Maruyama, T., A. McLachlan, S. Iino, K. Koike, K. Kurokawa, and D. R. Milich.** 1993. The serology of chronic hepatitis B infection revisited. J. Clin. Invest. **91:**2586–2595.
16. **Maruyama, T., F. Schodel, S. Iino, K. Koike, K. Yasuda, D. Peterson, and D. R. Milich.** 1994. Distinguishing between acute and symptomatic chronic hepatitis B virus infection. Gastroenterology **106:**1006–1015.
17. **Michel, M. L., H. Davis, M. Schleff, M. Mancini, P. Tiollais, and R. G. Wahlen.** 1995. DNA-mediated immunization to the hepatitis B surface antigen in mice: aspects of the humoral response mimic hepatitis B viral infection in humans. Proc. Natl. Acad. Sci. USA **92:**5307–5311.
18. **Milich, D. R., J. E. Jones, J. L. Hughes, T. Maruyama, J. Price, I. Melhado, and F. Jirik.** 1994. Extrathymic expression of the intracellular hepatitis B core antigen results in T cell tolerance in transgenic mice. J. Immunol. **152:**455–466.
19. **Milich, D. R., J. E. Jones, J. L. Hughes, J. Price, A. K. Raney, and A. McLachlan.** 1990. Is a function of the secreted hepatitis B e antigen to induce immunologic tolerance in utero? Proc. Natl. Acad. Sci. USA **87:**6599–6603.
20. **Milich, D. R., and A. McLachlan.** 1986. The nucleocapsid of the hepatitis B virus is both a T-cell dependent and a T-cell independent antigen. Science **234:**1398–1401.
21. **Milich, D. R., A. McLachlan, A. K. Raney, R. Houghten, G. B. Thornton, T. Maruyama, J. L. Hughes, and J. E. Jones.** 1991. Autoantibody production in hepatitis B e antigen transgenic mice elicited with a self T-cell peptide and inhibited with nonself peptides. Proc. Natl. Acad. Sci. USA **88:**4348–4352.
22. **Milich, D. R., A. McLachlan, S. Stahl, P. Wingfield, G. B. Thornton, J. L. Hughes, and J. E. Jones.** 1988. Comparative immunogenicity of hepatitis B virus core and e antigens. J. Immunol. **141:**3617–3624.
23. **Milich, D. R., A. McLachlan, G. B. Thornton, and J. L. Hughes.** 1987. Immune response to hepatitis B virus core antigen HBcAg: localization of T cell recognition sites within HBcAg/HBeAg. J. Immunol. **139:**1223–1231.
24. **Milich, D. R., D. L. Peterson, F. Schödel, J. E. Jones, and J. L. Hughes.** 1995. Preferential recognition of the hepatitis B nucleocapsid antigens by Th_1 or Th_2 cells is epitope and major histocompatibility complex dependent. J. Virol. **69:**2776–2785.
25. **Milich, D. R., F. Schödel, D. L. Peterson, J. Jones, and J. L. Hughes.** 1995. Characterization of self-reactive T cells that evade tolerance in hepatitis B e antigen transgenic mice. Eur. J. Immunol. **25:**1663–1672.
26. **Milich, D. R., S. F. Wolf, J. L. Hughes, and J. J. Jones.** 1995. Interleukin 12 suppresses autoantibody production by reversing helper T-cell phenotype in hepatitis B e antigen transgenic mice. Proc. Natl. Acad. Sci. USA **92:**6847–6851.
27. **Ou, J., O. Laub, and W. Rutter.** 1986. Hepatitis B virus gene function: the precore region targets the core antigen to cellular membranes and causes the secretion of the e antigen. Proc. Natl. Acad. Sci. USA **83:**1578–1582.
28. **Petit, M.-A., and J. Pillot.** 1985. HBc and HBe antigenicity and DNA-binding activity of major core protein P22 in hepatitis B virus core particles isolated from the cytoplasm of human liver cells. J. Virol. **53:**543–551.
29. **Pushko, P., M. Sällberg, G. Borisova, U. Ruden, V. Bichko, B. Wahren, P. Pumpens, and L. Magnius.** 1994. Identification of hepatitis B virus core protein regions exposed or internalized at the surface of HBcAg particles by scanning with monoclonal antibodies. Virology **202:**912–920.
30. **Raz, E., H. Tighe, Y. Sato, M. Corr, J. A. Dudler, M. Roman, S. L. Swain, H. L. Spiegelberg, and D. A. Carson.** 1996. Preferential induction of a Th-1 immune response and inhibition of specific IgE antibody formation by plas-

mid DNA immunization. Proc. Natl. Acad. Sci. USA **93:**5141–5145.
31. **Salfeld, J., E. Pfaff, M. Noah, and H. Schaller.** 1989. Antigenic determinants and functional domains in core antigen and e antigen from hepatitis B virus. J. Virol. **63:**798–808.
32. **Sällberg, M., P. Pushko, I. Berzinsh, V. Bichko, P. Sillekens, M. Noah, P. Pumpens, E. Grens, B. Wahren, and L. O. Magnius.** 1993. Immunochemical structure of the carboxy-terminal part of hepatitis B e antigen: identification of internal and surface-exposed sequences. J. Gen. Virol. **74:**1335–1340.
33. **Sällberg, M., U. Ruden, L. O. Magnius, E. Norrby, and B. Wahren.** 1991. Rapid "tea-bag" peptide synthesis using 9-fluorenylmethoxycarbonyl (Fmoc) protected amino acids applied for antigenic mapping of viral proteins. Immunol. Lett. **30:**59–68.
34. **Sällberg, M., U. Ruden, B. Wahren, M. Noah, and L. O. Magnius.** 1991. Human and murine B-cells recognize the HBeAg/beta (or HBe2) epitope as a linear determinant. Mol. Immunol. **28:**719–726.
35. **Schirmbeck, R., W. Böhm, K. Ando, F. V. Chisari, and J. Reimann.** 1995. Nucleic acid vaccination primes hepatitis B virus surface antigen-specific cytotoxic T lymphocytes in nonresponder mice. J. Virol. **69:**5929–5934.
36. **Schirmbeck, R., K. Melber, A. Kuhnröber, Z. A. Janowicz, and J. Reimann.** 1994. Immunization with soluble hepatitis B surface (S) protein particles elicits murine H-2 class I-restricted CD8+ cytotoxic T lymphocyte responses in vivo. J. Immunol. **152:**1110–1119.
37. **Schodel, F., D. Peterson, J. Zheng, J. E. Jones, J. L. Hughes, and D. R. Milich.** 1993. Structure of hepatitis B virus core and e-antigen. A single precore amino acid prevents nucleocapsid assembly. J. Biol. Chem. **268:** 1332–1337.
38. **Stevens, T. L., A. Bossie, V. M. Sanders, R. Fernandez-Botran, R. L. Coffman, T. R. Mossman, and E. S. Vitetta.** 1988. Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. Nature **334:**255–258.
39. **Tiollais, P., C. Pourcel, and A. Dejean.** 1985. The hepatitis B virus. Nature **317:**489–495.
40. **Townsend, K., M. Sällberg, J. O'Dea, T. Banks, D. Drive, S. Sauter, S. M. Chang, D. J. Jolly, S. J. Mento, D. R. Milich, and W. T. L. Lee.** 1997. Characterization of CD8+ cytotoxic T-lymphocyte responses after genetic immunization with retrovirus vectors expressing different forms of the hepatitis B virus core and e antigens. J. Virol. **71:**3365–3374.
41. **Tsai, S. L., P. L. Chen, M. Y. Lai, P. M. Yang, J. L. Sung, J. H. Huang, and L. H. Hwang.** 1992. Acute exacerbations of chronic type B hepatitis are accompanied by increased T cell responses to hepatitis B core and e antigens. J. Clin. Invest. **89:**87–96.